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14. ABSTRACT
We examined the concept that individuals vary in their propensity to methylate promoter CpG islands by measuring such methylation in their peripheral blood. Individuals more prone to methylate CpG islands may be more susceptible to breast or other cancers. Scope: Our objective was to test peripheral blood for promoter methylation of selected genes known to become methylated in breast cancer using sensitive and specific quantitative assays. Major findings/ up-to-date report of the progress in terms of results and significance: We developed several powerful new methodologies based on high resolution melting for sensitive detection of DNA methylation and to genotype DNA for SNPS potentially involved in methylation. Promoter region methylation was observed in all tested blood DNA samples for *CDH1* and *HIC1,* in the majority of DNA samples for *TWIST1* and *DAPK1* and in a substantial proportion of the DNA samples for *MGMT*, *APC* and *RARB*. *MGMT* methylation was associated with a single nucleotide polymorphism. No *BRCA1*, *CDKN2A*, *GSTP1* or *RASSF1A* promoter methylation was found in this sample set. Several individuals had higher levels of methylation at several loci suggestive of a methylator phenotype. These findings will be implemented to establish case control studies to determine breast cancer risk.

15. SUBJECT TERMS
methylator in normal tissues, tumour suppressor genes, breast cancer predisposition

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INTRODUCTION:

DNA methylation changes are critical in breast cancer development and progression. We examined the concept that individuals vary in their propensity to methylate promoter CpG islands. Assaying readily obtainable peripheral blood to identify individuals more prone to methylate their DNA, either at multiple loci or at specific driver tumor suppressor genes, may allow the determination of an individual's susceptibility to breast cancer. This may lead to a better understanding of how breast cancer develops and consequently how prevention strategies might be implemented. Methylation can be modified by diet and drugs and thus chemo-preventive measures may be of use in at risk individuals.

BODY:

Task 1: Determine the incidence of somatic promoter region methylation.

Our objective was to test peripheral blood for promoter methylation of genes known to be methylated in breast cancer using sensitive quantitative assays, to determine an index of methylation in somatic tissues, and to relate this to breast cancer predisposition. We also aimed to determine whether individual differences in methylation of normal tissues are related to polymorphisms in genes involved in the control of methyl metabolism.

Two methodologies were developed during the course of this study to accurately assess low levels of methylation. The first was methylation-sensitive high resolution melting which amplified sequences regardless of their methylation status and then used high resolution melting to differentiate between fully, partially and unmethylated sequences (Wojdacz & Dobrovic, 2007). The second methodology was SMART-MSP, a new quantitative variant of methylation specific PCR (MSP) that was able to identify false positive results (Kristensen et al, 2008) .

Although methylation-sensitive high resolution melting assay could be sensitive to as low as 0.1% methylation, heterogeneous methylation reduced the effective sensitivity of the assay. SMART-MSP proved to be routinely sensitive to as low as 0.1% methylation. We believe that SMART-MSP is superior to MethyLight and is particularly adapted to the requirements of this project.

A correlation between methylation of the *MGMT* promoter and the T allele of the rs16906252 single nucleotide polymorphism (SNP) located in the promoter was previously reported for carcinomas. As aberrant MGMT methylation can be an early event in tumor development, we tested the hypothesis that normal individuals possessing the T allele may be predisposed to somatic methylation at the MGMT promoter (Candiloro & Dobrovic, 2009). SMART-MSP was used to study methylation of the CpG island. Peripheral blood mononononuclear cell DNA from 89 normal, healthy individuals was genotyped at rs1690625 and assessed for the methylation status of the MGMT promoter region. There was a strong association between presence of the T allele and detectable methylation (P = 0.00005) in the peripheral blood DNA. Furthermore, when a MSP assay flanking the SNP was used to amplify methylated sequences in hetero-zygotes, only the T allele was methylated. Thus, detectable somatic methylation of the MGMT promoter in normal individuals is strongly associated with the T allele of the rs16906252 MGMT promoter SNP. This was the first report of A SNP influencing methylation in normal tissues and as such underlies the important of sequence changes in influencing methylation predisposition (Candiloro & Dobrovic, 2009). This publication was considered important enough to get an editorial in the same issue.

In the next study, DNA from 48 samples of normal peripheral blood mononuclear cells was evaluated for the presence of methylation of a panel of DNA methylation biomarkers that have been implicated in breast cancer (Kristensen et al, 2012). SMART-MSP enabled quantitative detection of low levels of methylated DNA. Methylation was observed in all tested mononuclear cell DNA samples for the *CDH1* and *HIC1* promoters and in majority of DNA samples for the *TWIST1* and *DAPK1* promoters. *APC* and *RARB* promoter methylation, at a lower average level, was also detected in a substantial proportion of DNA samples. We found no *BRCA1*, *CDKN2A*, *GSTP1* and *RASSF1A* promoter methylation in this sample set. Several individuals had higher levels of methylation at several loci suggestive of a methylator phenotype (Kristensen et al, 2012).

Conclusions: Our studies differ from other studies in that we take a clearly uninvolved tissue, peripheral blood leukocytes in order to determine the inherent epigenetic instability of an individual. Our results show that using sensitive assays that low level methylation is detectable for many genes. This can be a possible biomarker for breast cancer risk. The sensitive methylation assays developed would also be of use for monitoring the response of breast cancer to therapy. Furthermore, as methylation of some potential DNA methylation biomarkers can be detected in normal peripheral blood mononuclear cells, this is likely to affect their specificity for detecting low level disease. However, we found no evidence of promoter methylation for other genes indicating that panels of analytically sensitive and specific methylation biomarkers in body fluids can be obtained.

Publications deriving from Task 1:

Wojdacz T, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM) for rapid and sensitive assessment of methylation. Nucleic Acids Research 2007 35:e41 (7 pages; 129 citations till present)

Kristensen, LS, Mikeska T, Krypuy M, Dobrovic A. Sensitive Melting Analysis after Real Time-Methylation Sensitive PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. Nucleic Acids Research 2008 36:e42 (13 pages; 44 citations till present)

Candiloro IL, Dobrovic A. Detection of MGMT promoter methylation in normal individuals is strongly associated with the T allele of the rs16906252 MGMT promoter single nucleotide polymorphism. Cancer Prev Res. 2009 2:862-7. (6 pages; 25 citations till present)

Kristensen LS, Raynor M, Candiloro IL, Dobrovic A. Methylation profiling of normal individuals reveals mosaic promoter methylation of cancer associated genes. Oncotarget. 2012 3:450-61 (12 pages; 0 citations till present)

Task 2. Relate methylation propensity to enzyme polymorphisms

A third methodology based on high resolution melting was developed to genotype DNA samples for single nucleotide polymorphisms in several key genes that are involved in methyl metabolism and may directly or indirectly affect the methylation status of the DNA. High-resolution melting (HRM) shows great promise for high-throughput, rapid genotyping of individual polymorphic loci and this study showed that it was readily adaptable to a wide variety of loci. The following assays were designed and validated using high resolution melting assays; 5,10-methylenetetrahydrofolate reductase (*MTHFR*; C677T and A1298C), methionine synthetase/5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*; A2756G), and DNA methyltransferase 3b (*DNMT3b*; C46359T and C31721T) Analysis of the data found that none of the genotypes relates to the somatic methylation observed (unpublished data).

Publication deriving from Task 2: Kristensen LS, Dobrovic A. Direct genotyping of single nucleotide polymorphisms in methyl metabolism genes using probe free high resolution melting analysis. Cancer, Epidemiology, Biomarkers and Prevention 2008 17:1240-7. (8 pages; 25 citations till present)

Task 3. Relate methylation propensity to breast cancer predisposition

We are currently examining the influence of propensity to methylation on breast cancer risk in a case-control study. Various issues precluded the availability of the sample set during the initial funded period. However, this work is still being undertaken and will lead to a publication when completed. Related publications (funded by a separate DOD grant) have shown that detectable methylation of the *BRCA1* gene in the peripheral blood predisposes to breast tumours that are methylated at the *BRCA1* locus.

Publications related to Task 3:

Snell C, Krypuy M, Wong EM; kConFab investigators, Loughrey MB, Dobrovic A. BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumour phenotype. Breast Cancer Res. 2008;10:R12.

Wong EM, Southey MC, Fox SB, Brown MA, Dowty JG, Jenkins MA, Giles GG, Hopper JL, Dobrovic A. Constitutional methylation of the BRCA1 promoter is specifically associated with BRCA1 mutation-associated pathology in early-onset breast cancer. Cancer Prev Res (Phila). 2011 4:23-33.

Leveraging:

Funding from this project has enabled us to make substantial contributions to the literature and also provided the basis for a subsequent related grant.

In terms of publications, this includes two important review articles.

Dobrovic A, Kristensen LS. DNA methylation, epimutations and cancer predisposition. Int J Biochem Cell Biol. 2009 41:34-39. (6 pages; 28 citations till present)

Mikeska T, Candiloro I, Dobrovic A. The methodological implications of heterogeneous DNA methylation for the use of methylation as a biomarker. Epigenomics 2010 2:561-73 (13 pages; 19 citations till present)

An important set of methodology articles to which the funding indirectly contributed have also appeared**.**

Wojdacz TK, Hansen LL, Dobrovic A. A new approach to primer design for the control of PCR bias in methylation studies. BMC Res Notes. BMC Research Notes 2008, 1:54 (3 pages; 33 citations till present)

Wojdacz TK, Dobrovic A. Melting curve assays for DNA methylation analysis. Methods Mol Biol. 2009;507:229-40 (12 pages; 6 citations till present).

Wojdacz TK, Dobrovic A. Hansen LL. Methylation-sensitive high-resolution melting, Nature Protoc. 2008;3:1903-8 (6 pages; 43 citations till present)

The grant related to somatic predisposition was obtained from the Cancer Council of Victoria.

A new approach to identifying individuals at risk of cancer. Dobrovic A (2009-2011).

KEY RESEARCH ACCOMPLISHMENTS:

- Development of new methodology (MS-HRM) to detect low level methylation and publication of this methodology (Wojdacz & Dobrovic 2007)
- Development of methodology (SMART-MSP) to detect low level methylation and publication of this methodology (Kristensen et al. 2008)
- Development of methodology for rapid genotyping assays (Kristensen & Dobrovic, 2008)
- Identification of cancer associated genes that can be methylated in normal tissues (Kristensen et al, 2012).
- Demonstration that a promoter region SNP can predispose to methylation of that promoter in normal tissues (Candiloro & Dobrovic, 2009).
- Identification of genes that are methylated in cancer that are not normally methylated at detectable levels in normal tissues (Kristensen et al, 2012).

REPORTABLE OUTCOMES:

Multiple published manuscripts (listed both above and in the appendices below)

Results from this research was presented at multiple national and international meetings

American Association for Cancer Research, Annual Meeting, Los Angeles, April 2007.
Tomasz K. Wojdacz, Ee Ming Wong, Michael Krypuy, Alexander Dobrovic. High resolution melting allows sensitive high-throughput assessment of methylation in tumour samples

AACR meeting on Cancer Epigenetics, Boston, May 2008
Lasse S Kristensen, Michael Raynor and Alexander Dobrovic. Profiling of methylation in peripheral blood mononuclear cells by Sensitive Melting Analysis after Real Time - Methylation Specific PCR (SMART-MSP)

DOD Era of Hope, Baltimore, June 2008.
Lasse Kristensen, Michael Raynor and Alexander Dobrovic. Peripheral Blood Methylation: a Possible Biomarker for Predicting Breast Cancer Risk.

Cancer Epigenetics, Wilson's Prom, October 2008
"Somatic methylation and cancer predisposition (plenary oral)."

Australian Health and Medical Research Congress, Brisbane, Nov 2008
Alexander Dobrovic. "High resolution melting; a SMART way to study methylation." (oral).

Epigenetics 2009 Melbourne, December 1-4, 2009
Dobrovic A. Epigenetic and Genetic Origins of Human Cancer (plenary oral).

ILM Candiloro, A Dobrovic. DNA methylation at the MGMT promoter is associated with the T allele of the rs16906252 SNP in peripheral blood of normal individuals.

3rd Novartis Research & Development Symposium, Melbourne, May 1-2, 2010.
Dobrovic A. DNA methylation as a cancer biomarker (invited speaker).

Developments in Real-Time PCR Research & molecular diagnostics, Göteborg, Sweden, May 31-June 2, 2010.
Dobrovic A. Sensitive and accurate measurement of low level methylation (invited speaker).

Familial Aspects of Cancer 2010, Research and Practice, Kingscliff, NSW, August 201
Dobrovic A. The role of DNA methylation in cancer predisposition (oral).

20th Princess Takamatsu Symposium, Tokyo, November 2010.
Dobrovic A. "Detection and significance of low level methylation in normal tissues." (invited speaker).

Next Generation Sequencing Asia Congress, Singapore, October 3-4, 2011.
Dobrovic A.The challenge of detecting clinically significant low levels of methylation (invited speaker)

Cambridge Healthtech Institute's 6th annual Sequencing Solutions (XGen Congress and Expo) San Diego, CA, March 7 – 8, 2012.
Dobrovic A. The analysis of heterogeneous DNA methylation (invited).

CONCLUSIONS:

We have developed high-through put sensitive assays for the detection of methylated promoter regions from biopsy samples and blood. Low level methylation of some of the tested genes was observed in peripheral blood. The relationship of such methylation to breast cancer predisposition is the subject of continuing studies.

REFERENCES:

Not applicable

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

Tomasz Wojdacz M.Sc (University of Aarhus)
Lasse Kristensen M.Sc (University of Aarhus)

APPENDICES:

1. Publications arising from this funding. All these publications cite DOD funding. Those in bold represent those that are most closely aligned to the outcomes of this project. The first pages of the publications and the pages showing the attribution of the funding are attached. The complete publications could not be included due to document size limitations. They are available on request from the author.

Wojdacz T, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM) for rapid and sensitive assessment of methylation. Nucleic Acids Research 2007 35:e41
Kristensen, LS, Mikeska T, Krypuy M, Dobrovic A. Sensitive Melting Analysis after Real Time-Methylation Sensitive PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. Nucleic Acids Research 2008 36:e42
Kristensen LS, Dobrovic A. Direct genotyping of single nucleotide polymorphisms in methyl metabolism genes using probe free high resolution melting analysis. Cancer, Epidemiology, Biomarkers and Prevention 2008 17:1240-7.
Wojdacz TK, Hansen LL, Dobrovic A. A new approach to primer design for the control of PCR bias in methylation studies. BMC Res Notes. BMC Research Notes 2008, 1:54
Wojdacz TK, Dobrovic A. Melting curve assays for DNA methylation analysis. Methods Mol Biol. 2009;507:229-40.
Wojdacz TK, Dobrovic A. Hansen LL. Methylation-sensitive high-resolution melting, Nature Protoc. 2008;3:1903-8
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Candiloro IL, Dobrovic A. Detection of MGMT promoter methylation in normal individuals is strongly associated with the T allele of the rs16906252 MGMT promoter single nucleotide polymorphism. Cancer Prev Res. 2009 2:862-7.
Mikeska T, Candiloro I, Dobrovic A. The methodological implications of heterogeneous DNA methylation for the use of methylation as a biomarker. Epigenomics 2010 2:561-73 *
Kristensen LS, Raynor M, Candiloro IL, Dobrovic A. Methylation profiling of normal individuals reveals mosaic promoter methylation of cancer associated genes. Oncotarget. 2012 3:450-61

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Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation

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ABSTRACT

In this article, we show that high resolution melting analysis (HRM) is a sensitive and specific method for the detection of methylation. Methylated DNA and unmethylated DNA acquire different sequences after bisulphite treatment resulting in PCR products with markedly different melting profiles. We used PCR to amplify both methylated and unmethylated sequences and assessed HRM for the determination of the methylation status of the *MGMT* promoter region. Reconstruction experiments showed that *MGMT* methylation could be detected at levels as low as 0.1%. Moreover, MS-HRM allows for estimation of the methylation level by comparing the melting profiles of unknown PCR products to the melting profiles of PCR products derived from standards with a known unmethylated to methylated template ratio. We used MS-HRM for the analysis of eight cell lines of known methylation status and a panel of colorectal cancer specimens. The simplicity and high reproducibility of the MS-HRM protocol makes MS-HRM the method of choice for methylation assessment in many diagnostic and research applications.

INTRODUCTION

Methylation of cytosines allows the encoding of epigenetic information directly onto the DNA. In the human genome, methylated cytosines are found in CpG dinucleotides whose palindromic nature allows for the maintenance of methylation patterns by DNA methyltransferases following semi-conservative replication of DNA. Regions of DNA with a relatively high CpG dinucleotide content are referred to as CpG islands (1). CpG islands are distributed in a non-random manner across the human genome and often span the promoter region and the first exon of protein coding genes. Methylation of individual promoter region CpG islands usually acts to turn off (silence) transcription by recruiting histone deacetylases thereby inducing the formation of inactive chromatin (2).

Promoter region methylation of genes, particularly those genes with pivotal functions in relation to tumour suppression, apoptosis and DNA repair is one of the hallmarks of cancer (2). Alterations of the pattern of DNA methylation are an early event in cancer and continue on through the evolution of the cancer. Furthermore, distinct tumour types often have characteristic signatures of methylated genes (3,4) and these can be used as markers for early detection and/or monitoring the progression of carcinogenesis. More importantly, the methylation of certain genes, in particular DNA repair genes, can cause sensitivity to specific chemotherapeutics and methylation of those genes can thereby act as a predictive marker if those chemotherapeutic agents are used (5).

The methylation status of the *MGMT* gene has been shown to be a predictive marker in various cancers treated with alkylating agents (6–8). The MGMT protein removes methyl/alkyl adducts from the O^6-position of guanine and therefore protects the cell from undergoing transition mutations. The tumour-specific methylation of the *MGMT* promoter and subsequent abolition of MGMT protein activity will render tumour cells susceptible to alkylating agents used in cancer chemotherapy. Consistent with this, the survival of patients whose tumour was methylated at the *MGMT* promoter was significantly longer than that of patients with tumours that did not show methylation of *MGMT* when those patients were treated with alkylating agents (6–8).

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shown for the the *BNIP3* locus. The sensitivity of MS-HRM allows for detection of even a very small fraction of methylated material which is of importance as tumour samples may contain a low proportion of methylated sequences due to the presence of significant amounts of normal tissue or heterogeneity of the tumour. Furthermore, the high reproducibility and cost effectiveness of HRM makes this method suitable for both research and diagnostic applications.

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Conflict of interest statement. None declared.

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Sensitive Melting Analysis after Real Time-Methylation Specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection

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ABSTRACT

DNA methylation changes that are recurrent in cancer have generated great interest as potential biomarkers for the early detection and monitoring of cancer. In such situations, essential information is missed if the methylation detection is purely qualitative. We describe a new probe-free quantitative methylation-specific PCR (MSP) assay that incorporates evaluation of the amplicon by high-resolution melting (HRM) analysis. Depending on amplicon design, different types of information can be obtained from the HRM analysis. Much of this information cannot be obtained by electrophoretic analysis. In particular, identification of false positives due to incomplete bisulphite conversion or false priming is possible. Heterogeneous methylation can also be distinguished from homogeneous methylation. As proof of principle, we have developed assays for the promoter regions of the *CDH1*, *DAPK1*, *CDKN2A* (*p16^{INK4a}*) and *RARB* genes. We show that highly accurate quantification is possible in the range from 100% to 0.1% methylated template when 25 ng of bisulphite-modified DNA is used as a template for PCR. We have named this new approach to quantitative methylation detection, Sensitive Melting Analysis after Real Time (SMART)-MSP.

INTRODUCTION

In mammalian cells, DNA methylation occurs almost exclusively at the carbon-5 position of cytosine residues within CpG dinucleotides. The CpG dinucleotide is distributed in a non-random fashion throughout the human genome. CpG-depleted regions are interspersed with CpG-rich sequences referred to as CpG islands (1). These islands are often located at promoter regions of protein encoding genes and tend to be unmethylated (2,3).

Aberrant DNA methylation patterns are one of the hallmarks of cancer. In most cancers, promoter hypermethylation correlates with gene silencing. This has been shown for a wide range of tumour suppressor genes including the genes studied here and reviewed in (2,3): the cell-cycle inhibitor gene *CDKN2A* (*p16^{INK4a}*), the pro-apoptotic death-associated protein kinase gene *DAPK1*, the cell-adhesion gene *CDH1* and the retinoic acid receptor gene *RARB*.

In cancer, methylation of some promoter CpG islands can be an early event, and thus the detection of methylation shows great promise as a biomarker for early detection (4–6). Conventional methods for cancer detection are in general not capable of finding pre-neoplastic and small malignant lesions, and are thus not suitable for early detection. Molecular biomarkers in body liquids such as blood, sputum or urine that allow detection and diagnosis of tumours at an early stage would be ideal. However, in these types of samples, tumour-derived material is hard to detect because of the presence of material from normal cells, and thus highly sensitive methods are needed (7). As one example, methylation of the *CDKN2A* promoter has been detected in the sputum of smokers up to 3 years before they are diagnosed with cancer (8). Detection of low level methylation also shows great potential in the molecular monitoring of established disease after therapy (4). This has already been shown to be feasible in various cancers using DNA derived from plasma or serum (9,10).

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amplified in the exact same proportions. Nevertheless, WGA product is excellent as a negative control, especially when looking at low level methylation.

MSP was originally made quantitative by the use of TaqMan probes (19–21), but quantification without probes using the dye SYBR Green has been reported (38–41). SYBR Green intercalation into double-stranded DNA has been shown to be markedly influenced by salt concentration, by dye/base pair ratios which are not constant during the PCR since more and more double-stranded DNA is generated through each cycle, and to show sequence specific binding (42). For this reason, the number of PCR cycles can influence melting curve analysis (43). Generally, some of the problems associated with SYBR Green can be minimized if high dye/base pair ratios are used. However, SYBR Green cannot be used at saturating concentrations without inhibiting the PCR. These problems have been markedly reduced by the introduction of a new generation of dyes (22).

It has previously been shown that melting analysis can discriminate methylated from unmethylated DNA (44,45). These assays were based on methylation-independent PCR (MIP) primers, and have not become widely used, presumably because of the technical limitations of reagents, instrumentation and data analysis software used at that time. Generally, methods utilizing MIP primers can be compromised by the PCR bias phenomenon (46), but this is not an issue when MSP primers are used.

Melting curve analysis has also been used in combination with the MSP methodology as an alternative to gel electrophoresis (47). This methodology did not provide quantitative data or information that cannot be provided by gel electrophoresis. Melting analysis of MSP products in the presence of SYBR green have also been used to detect primer dimers (38). This can be done by gel electrophoresis as well. This study did provide quantitative data, but these were much less accurate compared to what we have obtained with a dye that does not inhibit PCR.

SMART-MSP is complementary to our previously described methodology using HRM, methylation-sensitive HRM (MS-HRM) (48). SMART-MSP uses MSP primers and quantification is based on C_T values instead of melting curve comparisons. Also SMART-MSP can detect the amplification of incompletely converted DNA, and is generally more sensitive. However, the main advantage of SMART-MSP might be that each assay is performed at one annealing temperature; where as in MS-HRM, a range of different temperatures are needed for the sensitive screening of samples showing markedly different methylation levels. In MS-HRM, quantification is based on comparisons with melting profiles of a standard dilution series that needs to be included in every run. This is not necessary when performing SMART-MSP assays which quantify relative to a 100% methylated control and the amplification of a CpG-free control sequence. We are currently using MS-HRM to analyse samples where relatively high levels of methylation are expected whereas SMART-MSP comes into its own to detect low levels of methylation.

Compared to the MethyLight technology, SMART-MSP does not require expensive probes. However, quantification without probes is compromised by primer dimers and non-specific amplification (38). For this reason, there is a need for evaluation of the PCR product, which can be conveniently done with HRM analysis. We observed non-specific amplification from some of our controls when the assays were performed at lower annealing temperatures. These products melted differently, and could be identified as non-specific amplification using gel electrophoresis as well (data not shown). However, when the assays were performed at the optimized annealing temperature, no primer dimers or non-specific products were observed. Without probes, less optimization may be needed and assay design has become easier. The use of HRM can give information about the methylation status of CpGs between the primers, but most importantly, the HRM step can be used as a control to indicate amplification of incompletely modified sequences, false priming or non-specific products. Thus, SMART-MSP is less prone to false-positive results and overestimation of methylation levels. We have shown that the sensitivity of our assays is similar to what has been reported for MethyLight.

In conclusion, SMART-MSP has made quantitative MSP inexpensive, more accurate, and less prone to false positives. It is a closed-tube method based on a high-throughput methodology, and thus it might prove to be the method of choice for the assessment of DNA methylation in clinical samples, particularly when low levels of methylation need to be sensitively and accurately determined.

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Conflict of interest statement. A provisional patent has been filed for this methodology by the Peter MacCallum Cancer Centre.

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Detection of *MGMT* Promoter Methylation in Normal Individuals Is Strongly Associated with the T Allele of the rs16906252 *MGMT* Promoter Single Nucleotide Polymorphism

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Abstract Methylation of the CpG island in the *MGMT* promoter region is a frequent event in several cancer types including colorectal cancer, lung cancer, lymphoma, and glioblastoma. A correlation between methylation and the T allele of the rs16906252 single nucleotide polymorphism (SNP) in colorectal carcinomas has previously been reported. As aberrant *MGMT* methylation can be an early event in tumor development, we tested the hypothesis that normal individuals possessing the T allele may be predisposed to somatic methylation at the *MGMT* promoter. Peripheral blood mononononuclear cell DNA from 89 normal, healthy individuals was genotyped at rs1690625 and assessed for the methylation status of the *MGMT* promoter region using independent quantitative methodologies capable of detecting low-level methylation: MethyLight and Sensitive Melting Analysis after Real-time Methylation-Specific PCR (SMART-MSP). There was a strong association between presence of the T allele and detectable methylation (P = 0.00005) in the peripheral blood DNA. Furthermore, when a MSP assay flanking the SNP was used to amplify methylated sequences in heterozygotes, only the T allele was methylated. Thus, detectable somatic methylation of the *MGMT* promoter in normal individuals is strongly associated with the T allele of the rs16906252 *MGMT* promoter SNP.

Inactivation of tumor suppressor genes and DNA stability genes by promoter methylation is a common occurrence in human cancer. In some cases, epigenetic inactivation of one of these genes is likely to be the initiating event in cancer development (reviewed in ref. 1). Evidence for this is particularly strong for certain hereditary cancer genes where constitutional methylation of the gene predisposes to cancer development (2–4).

It is also likely that constitutional methylation of other genes not normally involved in heritable cancer can predispose to cancer. One of the most plausible candidates for a gene whose inactivation may initiate carcinogenesis is *MGMT*, which codes for a protein that removes alkyl adducts from the O6 position of guanine (5). Loss of MGMT function will give rise to a mutator phenotype, as alkylation damage from a variety of environmental sources is a common occurrence and as alkylated guanine is likely to mispair with thymine during DNA replication. *MGMT* methylation is commonly found in various cancers including colorectal cancers, gliomas, head and neck cancers, and lymphomas (6). *MGMT* promoter methylation has been used as a predictive marker in cancers; it indicates those individuals who are likely to respond to chemotherapy with alkylating agents (7, 8).

MGMT methylation may be an early or even predisposing event in colorectal cancer. Esteller et al. (9) reported that *MGMT* methylation was present in adenomas. Shen et al. (10) reported that *MGMT* methylation was found in apparently normal colonic tissue up to 10 cm from an *MGMT*-methylated colorectal cancer indicating that *MGMT* methylation can even be observed prior to any detectable change in morphology.

Ogino et al. (11) investigated whether single nucleotide polymorphisms (SNP) in the *MGMT* gene had an effect on methylation of *MGMT* in colorectal cancers. The T allele of a SNP within the 5′ untranslated region (UTR; rs16906252; c.-56 C>T) was strongly associated with promoter methylation. We reasoned that the T allele might also affect the propensity to methylate the *MGMT* promoter in normal individuals. We thus sought to determine if mosaic methylation at the *MGMT* promoter was present in a readily assayed tissue, the peripheral blood, and if this was associated with the T allele.

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Methylation profiling of normal individuals reveals mosaic promoter methylation of cancer-associated genes.

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ABSTRACT:

Epigenetic silencing by promoter methylation of genes associated with cancer initiation and progression is a hallmark of tumour cells. As a consequence, testing for DNA methylation biomarkers in plasma or other body fluids shows great promise for detection of malignancies at early stages and/or for monitoring response to treatment. However, DNA from normal leukocytes may contribute to the DNA in plasma and will affect biomarker specificity if there is any methylation in the leukocytes. DNA from 48 samples of normal peripheral blood mononuclear cells was evaluated for the presence of methylation of a panel of DNA methylation biomarkers that have been implicated in cancer. SMART-MSP, a methylation specific PCR (MSP) methodology based on real time PCR amplification, high-resolution melting and strategic primer design, enabled quantitative detection of low levels of methylated DNA. Methylation was observed in all tested mononuclear cell DNA samples for the *CDH1* and *HIC1* promoters and in the majority of DNA samples for the *TWIST1* and *DAPK1* promoters. *APC* and *RARB* promoter methylation, at a lower average level, was also detected in a substantial proportion of the DNA samples. We found no *BRCA1*, *CDKN2A*, *GSTP1* and *RASSF1A* promoter methylation in this sample set. Several individuals had higher levels of methylation at several loci suggestive of a methylator phenotype. In conclusion, methylation of many potential DNA methylation biomarkers can be detected in normal peripheral blood mononuclear cells, and is likely to affect their specificity for detecting low level disease. However, we found no evidence of promoter methylation for other genes indicating that panels of analytically sensitive and specific methylation biomarkers in body fluids can be obtained.

INTRODUCTION

Recent research has led to the understanding that, despite the importance of genetic change, epigenetic mechanisms are perhaps the predominant drivers of cancer [1-3]. In particular, inactivation of genes by *de novo* promoter methylation accompanied by global hypomethylation is the most common DNA lesion of cancer cells [2, 3].

The CpG dinucleotide is the principal unit of methylation in humans as its palindromic pairing with a CpG dinucleotide on the other strand enables the semiconservative replication of methylation by DNA methyltransferase 1. Regions of high CpG density

[12]. Standard dilution series of 100%, 10%, 1%, 0.1%, 0.05% and 0% methylation levels were prepared by diluting the fully methylated DNA into unmethylated DNA. DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). 500 ng of genomic DNA or WGA product was subjected to bisulphite conversion with the EpiTect® 96 Bisulphite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

SMART-MSP primer design

The amplicons were designed to allow the HRM analysis to assess the conversion status of non-CpG cytosines between the primers [12]. The primers overlaid a minimum of 2 CpG sites with one of the cytosines of a CpG site placed at or adjacent to the 3' end. Non-CpG cytosines were included in the primer sequences to select against incompletely converted sequences, and at least one of these was placed as close to the 3' end as possible. The primer sequences, genomic regions spanned, amplicon sizes and the annealing temperatures are found in Table 1. The primers for *DAPK1* and *CDKN2A* ($p16^{INK4a}$) were previously published [12].

PCR and HRM conditions for the SMART-MSP assays

PCR cycling and HRM analysis were performed on the Rotor-Gene 6000™ (Corbett Research, Sydney, Australia). SYTO® 9 was used as the intercalating dye (Life Technologies, Carlsbad, CA). The reaction mixtures consisted of 25 ng of bisulphite modified template (pre bisulfite conversion amount), 1x PCR buffer, 2.5 mmol/L $MgCl_2$ final (3 mmol/L in the *CDH1* assay), 200 nmol/L of each primer, 200 µmol/L of each dNTP, 5 µmol/L of SYTO 9, 0.5U of HotStarTaq (Qiagen) in a final volume of 20 µL.

The PCR comprised one cycle of 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, annealing at the appropriate temperature (Table 1) for 30 s, 72°C for 30 s, and one cycle of 95°C for 1 min. HRM was performed from 60°C to 90°C, with a temperature increase at the rate of 0.2°C per second for all assays. The annealing temperature was experimentally determined for each assay to ensure only methylated templates were amplified. For each assay, a standard dilution series was run to assess the quantitative accuracy and sensitivity. Fully methylated and fully unmethylated control (WGA product), unmodified control, and no template control were also included in every run. All samples were analysed in duplicate.

The CDH1 MethyLight assay

The same forward primer was used for the MethyLight assay as for the SMART-MSP assay. The reverse primer sequence was: 5'-cgctaattaactaaaaattcacctaccg-3'. The probe sequence was FAM-5'-ttcgcgttgttgattgg-3'-BHQ (IDT). The two CpG sites between the primers are covered by the probe. The reaction mixtures consisted of 25 ng of bisulphite modified DNA, 1x PCR buffer, 250 nmol/L of probe, 3 mmol/L $MgCl_2$, 200 nmol/L of each primer, 200 µmol/L of each dNTP, and 0.5U of HotStarTaq (Qiagen) (5U/µL) in a total volume of 20 µL. The PCR comprised one cycle of 95°C for 15 min, 45 cycles of 95°C for 20 s and 64°C for 40 s. PCR was performed on the Rotor-Gene 6000. All samples were analysed in duplicate. In some reactions the probe was omitted and the primers were used for SMART-MSP to allow assessment of the methylation status of the two CpG sites in the region of the probe by HRM analysis.

Real-Time PCR quantification

The *COL2A1* control assay amplifying a CpG free region was used to normalise for DNA input after bisulphite conversion in the real-time PCR quantification [12]. The relative $2^{(-\text{delta delta CT})}$ quantification approach [40] was used. The C_T value of the control *COL2A1* assay (Table 1) is subtracted from the C_T value of the target gene for the calibrator sample (the 100% methylated standard). For each sample, this value is then subtracted from the value resulting from the C_T value of the target gene minus the C_T value for the *COL2A1* control assay. For this approach to be valid, the amplification efficiencies of the target and the control must be approximately equal [40]. The take-off values (defined as the cycle at which the second derivative is at 20% of the maximum level) given by comparative quantification (using the Rotor-Gene 6000 Series Software, version 1.7.61) were used as C_T values in the calculations.

Sequencing

Sequencing was used to verify a higher melting temperature was due to incomplete conversion of some of the non-CpG cytosines in between the *APC* SMART-MSP primers. For this purpose, a second amplification was performed with m13 tagged *APC* SMART-MSP primers. These products were sequenced using BigDye terminator chemistry v3.1 on an ABI 3730 (Applied Biosystems).

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Direct Genotyping of Single Nucleotide Polymorphisms in Methyl Metabolism Genes Using Probe-Free High-Resolution Melting Analysis

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Abstract

High-resolution melting (HRM) shows great promise for high-throughput, rapid genotyping of individual polymorphic loci. We have developed HRM assays for genotyping single nucleotide polymorphisms (SNP) in several key genes that are involved in methyl metabolism and may directly or indirectly affect the methylation status of the DNA. The SNPs are in the *5,10-methylenetetrahydrofolate reductase* (*MTHFR*; C677T and A1298C), *methionine synthetase* (*MTR*; *5-methyltetrahydrofolate-homocysteine methyltransferase*; A2756G), and *DNA methyltransferase 3b* (*DNMT3b*; C46359T and C31721T) loci. The choice of short amplicons led to greater melting temperature (T_m) differences between the two homozygous genotypes, which allowed accurate genotyping without the use of probes or spiking with control DNA. In the case of *MTHFR*, there is a second rarer SNP (rs4846051) close to the A1298C SNP that may result in inaccurate genotyping. We masked this second SNP by placing the primer over it and choosing a base at the polymorphic position that was equally mismatched to both alleles. The HRM assays were done on HRM capable real-time PCR machines rather than stand-alone HRM machines. Monitoring the amplification allows ready identification of samples that may give rise to aberrant melting curves because of PCR abnormalities. We show that samples amplifying markedly late can give rise to shifted melting curves without alteration of shapes and potentially lead to misclassification of genotypes. In conclusion, rapid and high-throughput SNP analysis can be done with probe-free HRM if sufficient attention is paid to amplicon design and quality control to omit aberrantly amplifying samples. (Cancer Epidemiol Biomarkers Prev 2008;17(5):1240–7)

Introduction

Abnormal methylation patterns are one of the hallmarks of cancer. Methylation of CpG islands in the promoter region of many genes, including tumor suppressor genes such as the cell cycle inhibitor $p16^{INK4a}$, the DNA repair genes *BRCA1*, *MLH1*, and *MGMT*, and the p53 regulator $p14^{ARF}$, has been shown to shut down their expression (1, 2). It is still incompletely understood what underlies this alteration of methylation patterns and which susceptibility factors are involved. Much effort has been put into solving these questions, but they still remain largely unanswered.

Common variants in genes involved in the metabolism of the methyl group are likely candidates for the variation underlying propensity to methylation in normal tissues as well as in tumors (3, 4). A sufficient supply of the methyl group donor *S*-adenosyl methionine (SAM) is important to maintain a normal methylation pattern (5, 6). Because the polymorphisms of the *5,10-methylenetetrahydrofolate reductase* (*MTHFR*), *5-methyltetrahydrofolate-homocysteine methyltransferase* (*MTR*), and *DNA methyltransferase 3b* (*DNMT3b*) genes studied here either influence or are influenced by the levels of SAM, they are of particular interest, especially because they have been reported to modify the risk of getting different types of malignancies. However, further investigations are needed. Not all forms of cancers have been investigated in this regard, and some results need validation. Interestingly, some of the variants have been shown to be associated with an increased risk of getting some cancers and a decreased risk of getting others (7-9).

SAM is synthesized using dietary methionine or methionine generated from homocysteine. *MTR* methylates homocysteine to generate methionine and thus influences the cellular levels of SAM. *MTHFR* catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the carbon donor for the *de novo* synthesis of methionine (Fig. 1). The common germ-line variants of *MTHFR* studied here (677T and 1298C) are less active (10, 11), and this can lead to higher levels of homocysteine and a deficiency of methyl group donors. The same is likely to be true for the *MTR* 2756G allele, but so far no one has been able to express human *MTR* in active form at sufficient levels to evaluate the biochemical effects of this polymorphism (12, 13). *DNMT3b* uses SAM to transfer methyl groups to DNA and is both responsible for *de novo* and maintenance DNA methylation. Overactivity of this gene has been linked to methylation of tumor suppressor genes and

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Medicine in focus

DNA methylation, epimutations and cancer predisposition

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Single nucleotide polymorphisms

ABSTRACT

Hereditary cancer syndromes caused by germline mutations give rise to distinct spectra of cancers with characteristic clinico-pathological features. Many of these hereditary cancer genes are silenced by methylation in a similar spectrum of sporadic cancers. It is likely that the initiating event in some of those cases of sporadic cancer is the somatic epigenetic inactivation (epimutation) of the same hereditary cancer gene. Recently, it has been shown that epimutations of certain hereditary cancer genes can be constitutional i.e. present throughout the soma. These epimutations may be inherited or arise very early in the germline. The heritability of these epimutations is very low as in most cases they are erased by passage through the germline. In other cases, predisposition to epimutations rather than the epimutations themselves can be inherited. These cases are characterised by Mendelian inheritance and are likely to be associated with sequence variants. Other sequence variants and environmental influences may also affect methylation propensity at a global level.

1. Introduction

This review will assess the role of epimutations, in particular epimutations of hereditary cancer genes, in the development of cancer. It will consider the evidence that there are cases of cancer in which epimutations not only directly predispose to the cancer, but are also widespread through adjacent tissues and unrelated tissues indicating a soma-wide event.

Epigenetic silencing of tumour suppressor genes associated with promoter methylation in tandem with an overall global reduction in DNA methylation is considered to be a hallmark of cancer cells (Esteller, 2008). As promoter methylation can lead to silencing that is mitotically transmissible, the term "epimutation", was introduced for any heritable change such as methylation that did not affect the actual sequence of the DNA (Holliday, 1987).

It is important to clarify the terms "somatic", "constitutional" and "germline" used to describe epimutations in this review. We will endeavour to use the terms as tightly as possible within this review while acknowledging that they may be more loosely used in the literature.

By "somatic", we refer to any epimutations that are observed in the tumour. The somatic epimutation may also be present as a precursor lesion in the apparently non-cancerous tissue from which the tumour arises. The presence of methylation in adjacent normal tissues is often referred to as a field effect and indicates that the apparently normal tissue is clonally related to the malignant cells.

By "germline", we refer to an epimutation that is found in all cells of the body and for which there is conclusive evidence of transmission of an actual epigenetic mark from the previous generation. As germline epimutation is present in every cell in the body, the risk of developing cancer will be similar to that of an individual that carries a germline mutation. However, it is still controversial whether germline epimutations occur in humans (Chong et al., 2007; Horsthemke, 2007; Leung et al., 2007; Suter and Martin, 2007).

By "constitutional", we refer to an epimutation that is found in all tissues of the body. There may be no, or equivocal, evidence of transmission from the previous generation. The epimutations may have occurred very early in development. In some cases, constitutional epimutations may be mosaic, i.e. they are present in all tissues but not all cells in those tissues have the epimutation.

Germline epimutations are constitutional but not all constitutional mutations are germline. Germline and constitutional epimutations have the common property that the same allele is methylated in all tissues of the individual. Somatic epimutations may arise more than once and thereby different alleles may be affected. Any one of these types of epimutations may be the

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genotype and environment in determining the frequency of epimutations.

10. Conclusions

The study of methylation in somatic tissues enabling the initial steps of tumourigenesis is still at an early stage. Constitutional methylation at specific tumour suppressor genes clearly underlies some cases which phenocopy hereditary cancer. Depending on the prevalence of such altered methylation, this may have important implications for genetic testing and counseling. Very little is known about what underlies the alterations of methylation patterns found in cancer cells. Genetic variations may affect both general and locus specific methylation propensity and thus directly affect cancer predisposition.

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The implications of heterogeneous DNA methylation for the accurate quantification of methylation

DNA methylation based biomarkers have considerable potential for molecular diagnostics, both as tumor specific biomarkers for the early detection or post-therapeutic monitoring of cancer as well as prognostic and predictive biomarkers for therapeutic stratification. Particularly in the former, the accurate estimation of DNA methylation is of compelling importance. However, quantification of DNA methylation has many traps for the unwary, especially when heterogeneous methylation comprising multiple alleles with varied DNA methylation patterns (epialleles) is present. The frequent occurrence of heterogeneous methylation as distinct from a simple mixture of fully methylated and unmethylated alleles is generally not taken into account when DNA methylation is considered as a cancer biomarker. When heterogeneous DNA methylation is present, the proportion of methylated molecules is difficult to quantify without a method that allows the measurement of individual epialleles. In this article, we critically assess the methodologies frequently used to investigate DNA methylation, with an emphasis on the detection and measurement of heterogeneous DNA methylation. The adoption of digital approaches will enable the effective use of heterogeneous DNA methylation as a cancer biomarker.

KEYWORDS: biomarker ■ cancer ■ *CDKN2B* ■ digital PCR ■ high-resolution melting ■ *MGMT* ■ minimal residual disease ■ molecular diagnostics

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DNA methylation has been recognized to play an important role in developmental biology, aging and cancer etiology [1–5]. Many genes are deregulated by DNA methylation in cancer [6,7]. Aberrant DNA methylation associated with certain genes has attracted considerable interest as a potential biomarker for the early detection of disease onset, prognosis and choice of treatment, and the monitoring of disease after therapy [8–10].

In mammals, DNA methylation occurs principally at CpG dinucleotides. CpG dinucleotides are unevenly distributed throughout the genome and the majority are normally methylated [11]. Some regions of the genome show a high CpG density spanning hundreds to thousands of base pairs, and are termed CpG islands [12]. These CpG islands are often associated with the promoter regions of genes and are then generally unmethylated [13]. If the promoter CpG islands become methylated, either as part of a developmental or pathological process, this leads to the formation of a repressive chromatin complex and the gene is silenced [14].

Heterogeneous DNA methylation

DNA methylation is usually analyzed in the context of a PCR amplicon generated from bisulfite treated DNA. Each CpG position in each of the template molecules can be either unmethylated or methylated. For the amplicon, fully methylated means that all the tested CpG positions in the amplicon are methylated. Similarly, (fully) unmethylated means that all the CpG positions in the amplicon are unmethylated.

Methylation heterogeneity can arise at several levels. At the simplest level, it has been used to refer to a mixture of fully methylated and unmethylated alleles. A homogeneous mixture of cells may contain both unmethylated and fully methylated alleles, such as is the case for imprinted genes, such as *H19* [15]. Alternatively, a heterogeneous mixture of cells may comprise methylated and unmethylated alleles in varying proportions.

In this article, we will reserve the term heterogeneous methylation for the specific context where multiple alleles, which differ in the pattern of methylated and unmethylated CpG sites, are present. The term epialleles can be useful to describe these multiple alleles. Each unique pattern of DNA methylation for a given genomic sequence, including fully methylated and unmethylated, would comprise one of the possible epialleles that can exist in a sample.

Figure 1 shows all eight possible epialleles for a region comprising three CpG positions. It should be noted that it is impossible to distinguish the DNA methylation scenarios shown in Figure 1A & 1B by methodologies (e.g., pyrosequencing) that can quantify methylation at individual CpG sites.

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Ultimately, analysis at the single molecule level without the need of bisulfite treatment and/or PCR amplification may be the ideal scenario. This approach would allow the direct visualization of DNA methylation at single CpG resolution without the confounding issues of the degree of bisulfite conversion and of PCR bias.

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A new approach to primer design for the control of PCR bias in methylation studies

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Abstract

Primer design for PCR-based methylation analysis following bisulfite conversion of DNA is considerably more complex than primer design for regular PCR. The choice of the optimal primer set is critical to the performance and correct interpretation of the results. Most methodologies in methylation analysis utilize primers that theoretically amplify methylated and unmethylated templates at the same time. The proportional amplification of all templates is critical but difficult to achieve due to PCR bias favouring the amplification of the unmethylated template. The focus of this brief communication is to point out the important criteria needed for the successful choice of primers that will enable the control of PCR bias in bisulfite based methylation-screening protocols.

Findings

With the increased awareness of the central role of epigenetic mechanisms in development and cancer, many techniques for the analysis of DNA methylation have been developed [1,2]. The overwhelming majority of these techniques involve sodium bisulfite modification of the DNA template, followed by PCR amplification of the region of interest.

The primers for analysis of DNA methylation status either seek (i) to interrogate the methylation of the CpG sites within the primer binding site e.g. methylation specific PCR (MSP) primers or, (ii) to amplify the region of interest regardless of its methylation status allowing for post-PCR determination of the methylation of the region of interest e.g. methylation independent PCR (MIP) primers.

In MSP, assessment of the methylation status of a given locus is determined by the CpG sites within the primer sequence. It is thus important to include several CpG sites towards the 3' end of the primers to ensure specific binding and subsequent amplification of only methylated variants of the template. Careful evaluation of MSP primers has to be performed prior to analyses to assure the specificity and exclude over interpretation of results.

MIP primers are required for applications in which the determination of methylation status of the sequence of interest is performed after the PCR. The post-PCR determination of the methylation status of amplified sequences can be performed in various ways such as: sequencing [3,4], restriction digestion [5,6], DHPLC [7], single strand conformation analysis [8,9], melting curve analysis [10,11] or high resolution melting [12,13].

5. The selected primers should be further evaluated in regard to standard parameters for primer design e.g. secondary structure, primer dimer formation. To evaluate those primer features, tools for primer design like Amplify http://Engels.genetics.wisc.edu/amplify/ can be used.

To test the primer set for the extent of the bias at various annealing temperatures, we PCR amplify a range of dilutions of a fully methylated DNA template into unmethylated DNA. The proportion of methylated to unmethylated sequences in the PCR product can then be estimated by melting analysis and the choice of annealing temperature which allows correction for PCR bias can be empirically determined [10,12]. In addition, the primer set has to be tested with non-bisulphite treated DNA as a template to eliminate the possibility that it amplifies PCR products from unconverted DNA.

It has been generally recognised that MSP can give higher positivity for methylation than MIP based methods. This has been attributed to the higher sensitivity and tendency to false positives of MSP. What has been less generally recognised is that MIP based techniques using MIP primers designed according to the commonly followed guidelines may fail to detect methylation at biologically significant levels [10]. The discrepant results obtained between the two approaches has been reported [16].

In conclusion, primer design for methylation studies is a complex task for MIP based protocols. Careful design and subsequent optimisation of the primer set has to be performed and each primer set has to be treated individually. Optimisation has to address both the PCR cycling conditions and the components of the PCR reaction to choose the optimal protocol for high performance for a given primer pair. The PCR bias in MIP based experiments in our experience was the main problem compromising these analyses. The guidelines for primer design presented here should assist in the design of methylation detection experiments whenever MIP primers are used such as bisulfite sequencing and nearly all methylation screening protocols including MS-HRM.

Competing interests

The authors are co inventors on patent applications on aspects of the MS-HRM methodology. The patents have been filed for by University of Aarhus and Peter MacCallum Cancer Centre.

Authors' contributions

TKW performed the experiments lending to this publication under the supervision of LLH and AD. All authors were involved in writing of this manuscript.

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Chapter 17

Melting Curve Assays for DNA Methylation Analysis

Tomasz K. Wojdacz and Alexander Dobrovic

Abstract

The ability of sodium bisulfite to modify cytosines in a methylation-dependent manner allows the conservation of DNA methylation information during PCR amplification. PCR products amplified from bisulfite-modified DNA have significantly different base compositions according to whether they originate from methylated or unmethylated variants of the target template. Different base compositions give rise to different thermal properties of the PCR products. Hence, melting analysis of amplification products in methylation studies allows the determination of whether the PCR products originate from methylated or unmethylated templates. Here, we briefly review recent advances in methodologies based on melting analyses of PCR products derived from bisulfite-modified templates and provide a methodology for methylation-sensitive high-resolution melting.

Key words: Methylation, melting curve, sodium bisulfite, high-resolution melting, PCR bias, Methylation-sensitive high-resolution melting (MS-HRM).

1. Introduction

The introduction of bisulfite modification of genomic DNA enabled the general use of PCR amplification in methylation studies *(1)*. Sodium bisulfite deaminates unmethylated cytosines to uracils leaving 5-methylcytosines intact. As a consequence, methylated cytosines are amplified during the subsequent polymerase chain reaction (PCR) as cytosines whereas unmethylated cytosines are amplified as thymines. Hence, the base composition of the PCR product depends on the 5-methylcytosine content of the template.

The two complementary strands of DNA are held together by hydrogen bonds and stacking interactions. Dissociation of double-stranded DNA is known as DNA melting or denaturation

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at 95°C, re-annealed by fast cooling and held for 1 min at 75°C. The HRM analyses were performed in the temperature interval 70–95°C with 50 acquisitions/°C and the default fluorescence temperature gradient parameters selected by the instrument (*see* **Section 3.7**). **Figures 17.2** and **17.3** show an example of the analysis of the results.

8. Heterogeneously methylated templates can be observed in many amplifications. On derivative curves, these are characterized by a broader melting peak typically starting before the unmethylated peak and extending into the methylated peak area. This is due to the formation of heteroduplexes, between heterogeneously methylated templates. An important advantage of MS-HRM is that, unlike many other methods, it allows the detection of heterogeneous methylation.

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Methylation-sensitive high-resolution melting

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The base composition of PCR products derived from sodium bisulfite-modified templates is methylation dependent. Hence, methylated and unmethylated, PCR products show different melting profiles when subjected to thermal denaturation. The methylation-sensitive high-resolution melting (MS-HRM) protocol is based on the comparison of the melting profiles of PCR products from unknown samples with profiles specific for PCR products derived from methylated and unmethylated control DNAs. The protocol consists of PCR amplification of bisulfite-modified DNA with primers designed to proportionally amplify both methylated and unmethylated templates and subsequent high-resolution melting analysis of the PCR product. The MS-HRM protocol allows in-tube determination of the methylation status of the locus of interest following sodium bisulfite modification of template DNA in less than 3 h. Here, we provide a protocol for MS-HRM, which enables highly sensitive, labor- and cost-efficient single-locus methylation studies on the basis of DNA high-resolution melting technology.

INTRODUCTION

Epigenetics is the study of somatically heritable changes of gene expression that occur without a change in the primary DNA sequence. Covalent histone modifications and changes of DNA methylation are the most widely investigated epigenetic mechanisms altering gene expression.

The methylation of cytosines occurs typically in CpG dinucleotides. CpG sites are non-randomly distributed throughout the human genome, with higher concentrations in the promoter and the first exon of the protein-coding genes. The regions with relatively higher CpG dinucleotide content are referred to as CpG islands (CGI). Up to 60% of protein-coding genes contain CGIs in the promoter region, and the methylation status of promoter CGIs generally correlates inversely with the transcriptional status of the gene[1].

Hypermethylation of the promoter CGIs of tumor suppressor genes has been recognized as an alternative mechanism in Knudson's two-hit theory of tumor suppressor gene inactivation, and the methylation of specific genes has been correlated with the outcome of different cancer types[2,3]. Many methylation changes have been shown to be cancer type specific and occur very early in carcinogenesis[4]. Moreover, the presence of differentially methylated sequences in body fluids, e.g., plasma and sputum, has been shown to be detectable long before clinical manifestation of the neoplastic disease[5].

Methylation changes are thus potentially powerful prognostic and predictive markers in cancer diagnosis and treatment. Furthermore, the methylation changes at imprinted loci have been shown as causative factor of many imprinting disorders, e.g., Beckwith Wiedemann[6], and age-dependent changes of methylation in particular are increasingly being associated with the pathology of many disorders[7].

In conclusion, new labor- and cost-efficient technologies are needed to allow high-throughput assessment of single-locus methylation changes and introduction of methylation tests into diagnostic settings.

PCR-based protocols are most widely used in the investigation of single-locus methylation changes[8,9]. Because methylation marks are removed from genomic DNA by DNA polymerase and not replicated during PCR amplification, the DNA template has to be chemically modified with the use of sodium bisulfite to preserve methylation information before PCR amplification. Sodium bisulfite changes unmethylated cytosines into uracil, whereas 5-methylcytosines are resistant to this modification. Subsequent amplification of bisulfite-modified template results in different amplicons from methylated and unmethylated templates: a relatively GC-rich PCR amplicon originating from methylated templates where methylated cytosines are preserved, and a GC-poor amplicon originating from unmethylated templates where all the cytosines are changed into uracils.

The techniques utilizing PCR amplification of bisulfite-modified DNA can be divided into two groups depending on the PCR primers used. One group utilizes primers that specifically amplify methylated (or unmethylated) templates, e.g., methylation-specific PCR (MSP) or quantitative MSP[10,11]. The second group is based on primers that allow amplification of the template regardless of its methylation status for post-PCR methylation analyses and include bisulfite sequencing[12,13], restriction digestion[14], single-strand conformation analysis[15], melting curve analysis[16] and high-resolution melting[17]. The proportional amplification of methylated and unmethylated templates is critical for this group of analyses, and preferential amplification of one of the templates (PCR bias) can lead to misinterpretation of the results. PCR bias is sequence dependent and has been shown to lead to under-amplification of the sequences originating from methylated templates[18–20]. We have addressed the PCR bias issue in methylation studies by developing a new primer design system that enables compensation for PCR bias and significantly increases the sensitivity of high-resolution melting-based methylation detection (see Experimental design). Our new primer design protocol can be used as an alternative to previously published guidelines for primer design[13] when PCR bias is encountered in the experiments.

The use of melting analyses in methylation studies was first reported by Guldberg and colleagues[16]. The recent development of new generation of melting instrumentation (HRM-capable fluorimeters), fluorescent dye chemistries and the new approach to primer design allowed the development of MS-HRM[6,17].

High-resolution melting technology was initially developed for genotyping studies and is based on the comparison of the melting profiles of sequences that differ in base composition and has been

Solution. Optimize annealing temperature and Mg^{+2} concentration of the PCR amplification, and redesign primers to include limited number of CpGs (see Experimental design).

ANTICIPATED RESULTS

Derivative peaks

When the derivative peaks data analyses format is used, for each of the unmethylated and methylated reference samples, an unambiguous peak should be obtained. The unknown sample can be scored on the bases of the similarities to one of the two reference profiles. The samples containing PCR product derived from both methylated and unmethylated templates will display two peaks similar to the methylated and unmethylated references (**Fig. 2**).

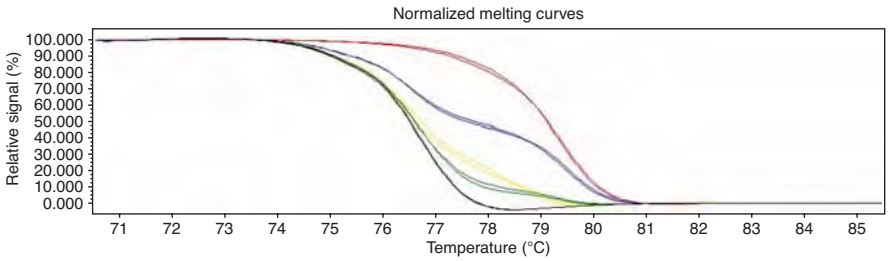

Figure 3 | Normalized HRM curve-based estimation of methylation levels for GSTP1 gene. The PCR product HRM curve derived from unknown sample (yellow) was plotted against HRM profiles of PCR product derived from standards with known concentration of methylated to unmethylated template. The results show that the methylation of the samples is in the range of 1–10% (100% red, 10% blue, 1% green, 0%/ unmethylated template black).

Normalized melting curves

Normalized HRM profiles allow estimation of methylation levels of unknown samples if they are run along with the standards representing different mixes of methylated and unmethylated templates. The methylation levels of an unknown sample can be estimated by comparing their melting profiles with the melting profiles of PCR products derived from controls with known methylated to unmethylated template ratio[17] (**Fig. 3**).

Heterogeneously methylated samples

A number of loci in the human genome do not undergo full methylation but are variably methylated at the CpGs, which is known as heterogeneous methylation. Therefore, the sequences that are heterogeneously methylated give rise to a mixture of PCR products with Ts at some CpG sites and Cs at others. The PCR products with minor differences in the sequence can cross-hybridize and form heteroduplexes. Heteroduplexes are less stable than homoduplexes in denaturing conditions and therefore display different melting temperature from the fully methylated and unmethylated references. The HRM melting profiles of the PCR products derived from the samples with heterogenously methylated templates show a characteristic complex melting pattern, which allows for their ready identification, especially when first derivative curves are analyzed. The heterogeneous pattern of methylation can be investigated in detail by sequencing-based methodologies.

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